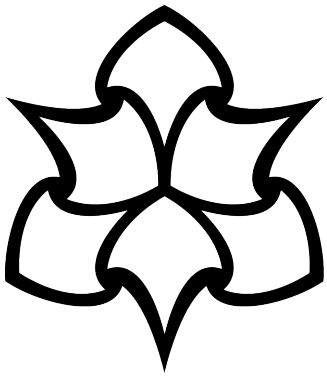

The influence of odour, taste and nutrients on feeding behaviour and food preferences in horses

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Abstract

While it has been established that nutrients and flavours (odour, taste) play an important role in diet selection by horses, previous studies have not always clarified what type of flavouring (e.g. non-nutritive or nutritive) was used. Therefore, the objective of this study was to determine the influence of distinct food characteristics (odour, taste, nutrients) on the preference of horses using different preference testing protocols. This experiment consisted of three phases; adaptation (P1), two-choice testing (P2) and multiple-choice testing using a chequerboard design (P3). Four pelleted diets equal in digestible energy, but contrasted in crude protein (LP; 14% and HP; 27%) and added non-caloric (natural) sweetener (i.e. LP, LP+, HP, HP+) were consecutively fed to each of sixteen adult horses. The diets were paired with four non-nutritive odours (coconut, banana, cinnamon, spearmint), with a unique odour and diet combination allocated to each group of four horses. In P1, each diet was presented solely for five days to facilitate pre- and post-ingestive associations; in P2 a two-choice test was conducted with four diet combinations (contrasts) over three days; and in P3 the four diets were presented simultaneously in a checkerboard fashion over a 5-day period. Feed intake, bucket/zone visits and time spent foraging or moving were recorded. The key findings of this study were: (1) In P1 an initially large variation in intake was recorded with only some horses showing a neophobic response to a new odour/food, but variation declined within 2 days with the majority of the horses consuming over 90% of the diets. (2) Nutrient (HP) content appeared to be the main driver for diet intake in P2 ($P<0.05$) and P3 ($P<0.001$). (3) Taste appeared to be the secondary determinant of preference and this was more evident with the LP diet. (4) Consumption of diets linked to sweet aromatic odours (banana and coconut) was greater in P3 ($P<0.001$). (5) The multiple-choice test, which was designed to promoted patch foraging behaviour, showed more explicit differences in diet ranking compared to the two-choice test. These findings confirm previous studies that horses prioritise diets on

nutrients, but this is the first equine study that shows the positive influence of a non-caloric natural sweetener on diet choice. A non-nutritive sweet taste or odour appears to encourage diet intake by horses, but more research is needed that examines different sweeteners coupled with and without odour and/or dietary nutrients and its long-term effects on food intake.

Key words

Food intake, Horses, Multiple-choice Design, Natural Sweetener, Odour, Protein.

Introduction

Food choice is determined by a complex of factors that include food sensory characteristics (smell, taste and texture), as well as post-ingestive feedback (positive or negative) (Garcia, 1989; Provenza, 1995). Typically nutritional consequences influence food preferences and sensory characteristics regulate the discrimination between various food items as demonstrated in humans (Stubbs and Whybrow, 2004), rats (Sclafani and Ackroff, 2004), and ruminants (Provenza and Villalba, 2006). However, pre-ingestive stimuli have been shown to override post-ingestive signals in some cases and sensory characteristics can induce preferences in the absence of any immediate post-ingestive feedback (Gherardi and Black, 1991; Berthoud, 2004).

While the interactions between pre- and post-ingestive feedback on food intake and preferences have been extensively studied in ruminants (sheep, goats and cattle), less is known about hindgut fermenters such as horses. It has been established that horses can develop conditioned food aversions (Houpt et al., 1990) and preferences (Goodwin et al., 2005a; b) and also make associations based on the nutritional content of foods (Laut et al.,

1985; Cairns et al., 2002; Redgate et al., 2014), but other studies have reported that diet selection and intake are largely influenced by the organoleptic qualities of foods such as odour, taste, ease of prehension and texture and that nutrient content appeared to be a weak indicator (Dulphy et al., 1997; Cuddeford, 2005). These equivocal results may be associated with long gut transit time, which may results in different gut-brain feedback mechanisms and/or secondary plant compound detoxification compared to ruminants, but no studies have been done to evaluate this.

Odour profiling has been used to make predictions about horses' preferences for different hays based on positive correlations found between detectable volatiles and nutritive or physical traits (Pain and Revell, 2007; 2009). However, these reports also identified volatiles in the hay that negatively influenced the preference but were not linked to any measurable nutritive and physical traits. The authors suggest that this may be related to other plant characteristics such as plant secondary compounds that may affect the taste or gut fermentation. This is in accordance with our previous study, which showed that strong herbaceous volatiles from novel forages affected preference negatively, even though the food itself had a good nutritional profile (van den Berg et al., 2016a). This implies that diet selection cannot always be explained by nutrient composition and that orosensory cues may override choices based on nutrition.

While it has been recognised that olfaction plays an important role in diet selection by horses, less is known about the influence of taste. It appears that horses have a preference for sweet (sucrose) solutions over sour, bitter or salty (Randall et al., 1978; Danel and Merkies, 2009; Merkies and Bogart, 2013). However, the influence of taste on food intake of horses has not been clearly defined. Commercially used flavours can either be categorized as aromatic

(odour) and non-nutritive such as a non-caloric sweetener; or nutritive, which include a caloric sweetener. Goodwin et al. (2005a) showed that well-liked flavours can be used to encourage intake of an unpalatable supplement. However, it is unclear as to what type of flavouring was used and whether it only affected the smell or also impacted the taste. In another study Goodwin et al. (2005b) offered four concentrate diets simultaneously that contained a combination of odour cues (mint, carrot, herbs, garlic) and added taste cues (molasses and sweetened syrup), and demonstrated that horses mix diets, selecting from preferred and less preferred diets. However due to the combination of odours and tastes it is unclear which food cues were the main drivers for the choices observed. In addition, a combination of formulations with different mix of macronutrients was tested and so it was also not clear if there was an effect of nutritional content on the diet selection.

Therefore, to enhance our understanding of the roles of pre- and post-ingestive cues on food intake and preference by horses the following study was conducted to examine the influence of distinct food characteristics i.e. nutrients (post-ingestive feedback) and, non-caloric taste and odour on the voluntary intake and preferences by horses. Horses were first exposed to individual diets to learn about the characteristics and post-ingestive associations. This was followed by two different preference tests (two-choice and multiple choice) to investigate feeding behaviour and food preferences. The multiple-choice test was developed using a checkerboard design and we hypothesised that horses would display patch foraging behaviour selecting all available foods, and they would do this in a sequence ranking of food choices primarily based on nutrients, followed by taste and then odour.

Materials and methods

Animals & husbandry

The study was conducted using 16 healthy horses; 10 mares and 6 geldings that had been managed as two groups on the same property at the University of Queensland (UQ Equine Unit). The horses were between the ages of 4 and 15 years (mean; 9), weighing 516-602 kg (mean; 559) and were of Australian Stock Horse, Standardbred or Thoroughbred breeds. Horses initially were grazing pasture and had a Henneke's body condition score between 4.5 and 5.5 (moderately thin to moderately fleshy, Henneke et al., 1983). The management and feeding of horses was based on the UQ Equine Unit's usual practices and throughout the study period horses were managed on pasture with no additional supplementary feeding, other than the experimental test diets. The study was conducted between the months of April and May 2015.

Diets and flavours

Four pelleted diets were formulated with similar digestible energy (DE) content (mean; 12.6 ± SD.; 0.22 Megajoule (MJ)) but differing in crude protein (CP) levels (Low CP (LP); 14% and High CP (HP); 27%) and added sweetener (included or absent). The chemical analysis of the diets is presented in Table 1. The pelleted diets were manufactured at the University of New England. The low energy/fibre pellets comprised of soybean hulls, beet pulp, black sunflower seeds and corn. To contrast the CP levels a proportion of corn was replaced with corn gluten in the HP diet. A commercially sourced human-grade non-caloric natural sweetener (blend of erythritol and stevia; Natures Flavors Inc, Orange, CA, USA) was added at 2.25% to one choice of the LP and HP diets. Erythritol is 60–70% as sweet as sucrose (table sugar) (de Cock, 2012) and Stevia is 300 times sweeter than table sugar (Goyal et al., 2010), yet both are almost non-caloric; the commercial blend had a 1:1 sensation with table sugar. To our knowledge no equine studies are known that have tested sweeteners in horse diets, therefore

the inclusion of 2.25% sweetener was based on an equal sugar sensation as 5% cane molasses inclusion, which is a standard rate used in sweet feeds by horse feed companies (Pratt-Phillips and Lawrence, 2014). Cane molasses is about 45-50% sugar (Najafpour and Poi Shan, 2003).

The four pelleted diets were paired with one of four odours (banana, coconut, cinnamon and spearmint) and the combination was randomised based on horse groups (Table 2). Commercially sourced human-grade (non-caloric) food flavour emulsions (coconut, banana, spearmint and cinnamon; Natures Flavors Inc, Orange, CA, USA) were used to make up odour solutions. Each odour was selected from a different odour class to aid the contrast i.e. fruit flavour (banana), nut flavour (coconut), herb flavour (spearmint) and spice flavour (cinnamon). Between 1 and 10 ml was diluted in 500 ml water to create a distinctive odour that was detectable by human senses and accepted by horses. The dilution ratio was based on a pilot study with four horses that were not part of this study. The diluted odour solutions were stored in four marked spraying bottles and 2-5 ml was misted (based on two enclosed hand squeezes of the spraying nozzle) onto the diets before they were offered to the horses.

Experimental design

The study was conducted in three phases. Before commencing the experiment, 16 horses were allocated to one of the four groups (A, B, C, D) (Table 2). The grouping of horses was done to ensure that the experiment was able to test the hypothesis based on nutrient composition and avoid bias to one particular odour. Hence each of the four diets was linked to all possible odour combinations (Latin square 4 x 4). Each horse was paired with another of similar weight, age and sex before randomly allocating one horse from each pair to one of the four groups (Table 3). This resulted in 2 groups with 3 female horses and 1 male horse and 2

groups with 2 female horses and 2 male horses with an almost identical weight and age distribution.

During phase 1 (adaptation) all horses were offered four pelleted diets paired with one of the four odours according to their allocated group, over a period of 20 days. Each diet was presented solely for five consecutive days to allow horses to make an association between each of the four diets and its allocated odour. This monadic phase also ensured that all horses were primed by this dietary experience (regardless of previous experiences) and equalized diet acceptance (intake of 80% or more) over five days. In phase 2 a series of two-choice tests were conducted with four diet combinations (contrasts) over three consecutive days to determine preferences (Table 4). Finally, in phase 3 preferences were tested again using a multiple-choice model that utilised a chequerboard design over a period of five days. The timeline of the experiments is illustrated in Figure 1.

Testing procedures

For the duration of phases 1 and 2, horses were individually fed in a yard that was familiar to them with other horses in sight to prevent undesired behaviours. In phase 1, horses were presented their allocated diet (400 g) for 15 minutes on five consecutive days before switching to the next diet/odour pair. In phase 2, horses were presented with two food choices (2 x 200 g) simultaneously (5 min). All four contrast two-choice tests were conducted on the same day, and this was repeated over three consecutive days. Horses were tested in a sequential order and presented with two tests consecutive with a 10 minutes break between. After all horses were tested the remaining two tests were presented in a similar fashion. The combination of the consecutive tests was randomised daily. The diets were presented in feeding tubs of a similar colour that were labelled for each odour to avoid odour mixing.

These feeding tubs were placed in larger bins that were mounted on the yard railing and under a shelter. When two food choices were offered the buckets were 0.5 m apart and the position of the bucket changed randomly for each testing day. Horses had *ad libitum* access to water in their yards. On completion of testing horses were returned to pasture.

In phase 3 a barren testing area (12 m x 12 m) divided into 16 zones (2.5 m^2) was used for the multiple-choice test. There were four zones allocated to each diet option in a chequerboard fashion, which was adapted from our previous study (van den Berg et al., 2016a) (Figure 2). Each zone contained 100 g of one of the diets, which was offered in feeding tubs of a similar colour and placed in rubber tyres. To avoid odour mixing each feeding tub was labelled for odour (4 x 4) and used throughout the testing period. In addition, the rubber tyres were labelled with coloured tape corresponding to the odour to facilitate randomisation to zones. Rubber matting 1 x 1 m was placed under the feeding tubs and rubber tyres. Horses were individually led into the testing area by a handler and allowed 7.5 min to forage the area uninhibited. A longer testing period was selected to allow for exploration and movement time between zones/buckets. On every testing day the diets were randomly allocated to a new zone. There were group yards with companion animals on both sides of the testing area. Before the start of the experiment, horses were familiarised with the test area and the routine of leading them separately into the testing area (Figure 1). On completion of testing horses were returned to pasture.

Feeding and measurements

In phase 1, horses were fed the single diets in the morning between 08:30 to 09:30 h and the intake (g) recorded on each of the five days. In phase 2 the four two-choice contrast tests (5 min each) were conducted in two parts; morning (08:00 – 12:00 h) and afternoon (13:00-

17:00 h) and in phase 3 the multiple-choice test (7.5 min) was conducted between 8:00-12:00 h. Behaviours for phase 2 and 3 were recorded with two video recorders (Panasonic HC-V160, Panasonic Corporation, Kadoma, Osaka, Japan and GoPro Hero 3+, GoPro, San Mateo, CA, USA) and by a person sitting 10 m outside the testing arena (under a shelter construction). The number of visits to each bucket or zone (categorised as both front hooves being placed in a zone) and sequence to each zone/bucket were documented. In addition, the time spent foraging (labelled as standing and chewing) or moving to each zone/bucket (classified as walking towards a new zone/bucket) were recorded. The intake of foods by each horse was determined by weighing the foods in each feeding bucket before and after each test. The intake was adjusted for moisture and calculated to a dry matter (DM) basis.

Statistical analysis

Diet intake, bucket/zone visits and time spent foraging or moving were analysed in R Studio version 0.99.484 (Team, 2015) and all data were checked for normality (Q-Q plots and Shapiro-Wilk test) and transformed where necessary. For all tests the level of significance was set to 5%.

Phase 1: Adaptation

Feed intake of each diet over the four weeks was assessed to determine the acceptance of the diets and post-ingestive associations. We considered an intake of 80% (~ 300 g DM) as the threshold for diet acceptance, based on the identified plateau curve of feed intake. The intake of each diet (and week) was denoted as the proportion (%) consumed out of the total offered and were logit-transformed. However, due to the large variation between the animals in feed intake behaviour on the first and second day of the diet introduction none of the classical statistical models applied showed a correct fit. Therefore, descriptive analyses were

conducted and the variance between diets, odours, groups and days were examined using a Fligner-Killeen test of homogeneity of variances.

Phase 2: Two-choice contrast tests

To determine the diet preference of each two-choice test the intake ratio of lower (Bucket 1) to higher (Bucket 2) palatability contrast over a 3-day testing period was examined using a generalized linear model (GLM) with a binomial distribution. In the model day and group were included as factors; odour was left out of the model as it was coupled to the group. Similar GLM models were used for the ratios (Bucket 1: Bucket 2) of bucket visits and time spent foraging or moving towards the buckets. Additionally, the levels of the diets, odours and groups (independent variables-factors) for all tests and days of Phase 2 were ranked using three linear regression models having the intake (g, DM) as response variable.

Phase 3: Multiple-choice test

The intake (g, DM) of each diet over the 5-day testing period was examined using a linear regression model with diet, day, odour and group included as factors. A similar model was used for the time spent foraging. For the zone count a GLM model with a Poisson distribution was fitted with diet, day, odour and group as factors. For the time spent moving a similar GLM model was used with the same explanatory factors.

Results

Phase 1: Adaptation

The intake proportion (%) of the four diets consumed out of the total offered over five days is given in Figure 3. The Fligner-Killeen tests indicated a departure from homogeneity for the

population's variances of intake proportions between diets (P<0.001) and days (P<0.001). In week 1 (LP diet), a large variation in intake between horses was observed on Day 1 and 2 (from 0% to 100% ingestion), which declined over time with 12 out of 16 horses consuming 90% or more after Day 2 and by Day 5 all horses ingested 95-100% of the offered diet. In week 2 (LP+ diet) a greater variation was only observed during the first two days, with all horses consuming over 90% of the offered diet after Day 2. Similar patterns where observed for week 3 (HP diet), however one horse was below 90% intake on Day 4 only. In week 4 (HP+ diet), horses showed a stable intake (95-100%) over all days, with only one horse below 80% on Day 4 and one horse below 90% on Day 5. The decreasing pattern in variance over time was also observed when reviewing the intake proportions for each group and odour. However, the Fligner-Killeen tests indicated a departure from homogeneity for the population's variances of intake proportions for groups (P<0.001), whereas we cannot reject the null-hypothesis for odours (P=0.08); indicating an equality of variance. The plotted data of Group B and D showed a larger distribution of variance compared to Group A and C.

Phase 2: Two-choice contrast tests

The fitted parameters of the GLM (binomial) model to ratios of intake, bucket visits and time spent foraging or moving of lower (Bucket 1) to higher (Bucket 2) palatability contrast for the four two-choice tests are given in Table 5. Data is presented as log-transformed (± SE) and expected back-transformed (multiplicative) ratios. Expected back-transformed ratios are used for the interpretation of the results for each test.

Test 1: LP vs. LP+

Analysis of deviance using GLM models indicated a significant effect for days (P=0.02). The expected intake ratios were increased for Day 2 (x 1.09) and Day 3 (x1.11) compared to the

initial ratio (0.93). Groups did not contribute to the model at the 5% significance level (P=0.051). Similar results were found for the time spent foraging ratio, showing a significant contribution for day factor (deviance test; P<0.001). In addition, a significant group effect was recorded (deviance test; P<0.001). The expected ratio was decreased for Group B (x 0.81), showing that more time was spent foraging on the LP+ diet, compared to the initial ratio (0.92). For both the bucket visit and time spent moving ratios the analysis of deviance did not suggest a contribution for days and groups.

Test 2: LP vs. HP

For the intake ratios the day factor did not contribute to the model showing similar ratios across days. Only a significant contribution for groups (deviance test; P<0.001) was observed. The expected intake ratio was decreased for Group B (x 0.9), showing a greater preference for the HP diet, compared to the initial ratio (0.93). This was linked to a significant odour effect (deviance test; P<0.001), indicating a lower intake ratio for the diet linked to the cinnamon odour (i.e. LP diet for Group B). Comparable results for the time spent foraging were found, suggesting no effect for days. A significant contribution for groups (deviance test; P<0.001) was observed. The expected ratio was decreased for Group B (x 0.76) compared to the initial ratio (0.86), whereas the ratios for Group C (x 1.12) and D (x 1.05) were increased. Group A and Group B appeared to spend more time foraging on the HP diet. For both the time spent moving and bucket visit ratios the day and group factors did not contribute to the models.

Test 3: HP vs. HP+

The GLM model does not suggest a significant contribution for days and groups for the intake ratio. However, for time spent foraging day factor (deviance test; P<0.001) contributed to the model. The expected ratios were increased for Day 2 (x 1.28) and Day 3 (x 1.06) compared to

the initial ratio (0.9). In addition, a significant contribution for group factor (deviance test; P<0.001) was observed. The expected time spent foraging ratios were increased for Group C (x 1.15) and Group D (1.09) compared to the initial ratio (0.9). For both bucket visit and time spent moving ratios the analysis of deviance did not suggest a contribution for days and groups.

Test 4: LP+ vs. HP+

The analysis of deviance suggests that only the group factor (P=0.003) contributed to the model for the intake ratios. The expected intake ratio was decreased for Group B (x 0.86), showing a greater preference for the HP+ diet, compared to the initial ratio (0.99). This was linked to a significant odour effect (deviance test; P<0.001), indicating a lower intake ratio for the diet linked to the coconut odour (i.e. LP+ diet for Group B). The GLM model for the time spent foraging suggests a contribution for day (P<0.001). The expected ratio was decreased for Day 3 (x 0.79) compared to the initial ratio (1.19). There was also a significant group effect (P<0.001) recorded for the time spent foraging ratios. The expected ratios were decreased for Group B (x 0.64), Group C (x 0.88) and Group D (x 0.87), showing that more time was spent foraging on the HP+ diet, compared to the initial ratio (1.19). For both the bucket visits and time spent moving ratios the day and group factors did not contribute to the model.

Ranking

The rankings of the diets, odours and groups were based on the mean intake (g, DM) of all tests and days combined. A significantly lower mean intake was recorded for the LP diet (163.9) compared to the other diets with the highest consumption for the HP+ diet (177.0) (SE; ± 1.73; P<0.05). Mean intake of HP (171.1) and LP+ (169.6) diets did not significantly

differ. No significant differences between odours were recorded, showing a similar mean intake for spearmint (172.5), banana (171.5), coconut (169.9) and cinnamon (167.6) (SE; ± 1.78). The difference between cinnamon and spearmint approached significance (P=0.053). A significantly greater consumption was recorded for Group C (179.8) and D (178.6) compared to Group A (167.9), with Group B (155.2) showing the lowest mean intake (SE; ± 1.47; P<0.001).

Phase 3: Multiple-choice test

The fitted parameters of the Linear regression and GLM (Poisson) models to intake, zone count and time spent foraging or moving are given in Table 6. The fitted parameters of the GLM models are presented as log-transformed (± SE) and expected back-transformed means. Expected back-transformed means (multiplicative) are used for the interpretation of the time spent moving and zone count results.

Intake and time spent foraging

The ANOVA using linear models indicated a significant effect for diet, odour and group (P<0.001). The intercept of the model was 109.3 ± 15.0 g and comprised LP diet, Day 1, Group A and banana odour. A significantly lower mean intake (g) was observed for the LP diet compared to the other diets with the highest consumption for the HP+ diet (increase of 73.6 ± 11.3 g) (P<0.001). Mean diet intake increased with 40.3 ± 11.3 g for the LP+ diet and 41.5 ± 11.3 g for the HP diet, which did not differ significantly. No differences in mean intake between the days (P=0.52) were recorded but there was a significantly greater preference for banana odour compared to cinnamon (-34.7 ± 11.3 g) and spearmint odour (-55.0 ± 11.3 g) (P<0.001). A group difference was observed, with Group D (50.9 ± 11.3 g) and Group C

(45.8 ± 11.3 g) having a significantly higher intake compared to group A (P<0.001), but Group A did not differ from Group B.

A strong linear correlation between the intake and time spent foraging (r=0.80) was observed. The linear models suggested a significant effect for diet and odour (ANOVA; P<0.001). The intercept of the model was 89.6 ± 11.2 sec and comprised LP diet, Day 1, Group A and banana odour. In accordance with the intake, significantly less time was spent foraging (sec) on the LP diet compared to the other diets (P<0.001), and the greatest time spent foraging was observed for the HP+ diet (increase of 44.6 ± 8.5 sec). More time was spent foraging on diets linked to the banana odour compared to the other odours (P<0.001). No differences in mean time spent foraging were observed for the different days and groups.

Time moving and zone count

Whilst there was a high correlation between time spent moving and zone count (r=0.94), showing a very close agreement, we continued using the time spent moving and zone counts as dependent variables to the two GLM models. The analysis of deviance for time spent moving towards zones/buckets suggests a significant effect for diets (P=0.013), days (P=0.009), group (P<0.001) and odour (P<0.001). The expected mean for the intercept was 8.8 sec and comprised LP diet, Day 1, Group A and banana odour. The model indicated that horses spent more time moving towards HP (x 1.16) and HP+ (x 1.13) diets compared to LP diet, which did not differ from LP+ diet (x 1.01). Horses spent more time moving on Day 5 (x 1.18) compared to the other days. Group A spent more time moving towards zones/buckets compared to Group D (x 0.84) with the lowest time observed for Group B (x 0.61). In accordance with the intake and time spent foraging trends, less time was spent moving towards the diets with spearmint odour (x 0.77) compared to the other odours. The GLM

model suggests only a significant effect for groups on the zone count (deviance test; $P<0.001$). The expected mean for the intercept was 2.7 and comprised LP diet, Day 1, Group A and banana odour. Group B (x 0.62) made fewer zone visits compared to the other groups.

Discussion

We hypothesised that horses would display more distinct patch foraging behaviour in the multiple-choice model selecting all available foods, and that horses would rank preferences based on nutritional content, followed by taste then odour. The key findings of this study were: (1) An initial large variation in diet intake was observed in the adaptation phase with some horses showing a neophobic response while others exhibited no apparent recognition of the odour/food being new, but variances declined within 2 days with majority of the horses consuming over 90% of the diets. (2) Nutrient (HP) content appeared to be the main driver for diet selection and feed intake in both preference tests. (3) Taste appeared to be the secondary determinant for preference by horses and this was more evident with the lower CP diet. (4) A greater intake of diets linked to sweet aromatic odours (banana and coconut) was observed. (5) The multiple-choice test promoted patch foraging behaviour and showed more explicit differences in diet selection compared to the two-choice test. (6) A significant group effect for diet preference and total feed intake was recorded.

The influence of nutrients on diet selection

After the monadic phase the preferences for the four diets were initially evaluated in four contrast tests using a two-choice test. None of the models were able to demonstrate that horses had an obvious preference for diets with a greater palatability, showing a close to 1:1 intake ratio for most of the tests and days. Yet, some of the tests suggested that more time was spent foraging on the diets with enhanced palatability, showing a slight departure from a 1:1

ratio; which was not consistent for all test days. The discrepancy between the observations for intake and time spent foraging may be a result of the fact that a number of horses were able to empty both buckets before the 5 min time period had elapsed and subsequently continued visiting the buckets to try and obtain left-over pellets. Therefore some of the time spent foraging could have been searching rather than ingestive behaviour. In hindsight, the test time should have been 3.5-4 min. Nonetheless, the contrast test results and mean intake ranking of diets suggest that horses did discriminate based on the nutrient content and showed a preference for the higher CP diet. This difference was less evident when a sweetener was added to the diet, an observation supported by the mean intake measures showing a ranking based on protein content but there were no significant differences in intake for the LP+ and HP diets. A similar ranking was also recorded in the multiple-choice test and these findings are in accord with other studies that have reported that preferences and intake are linked to macronutrient content (Laut et al., 1985; Cairns et al., 2002; Goodwin et al., 2005a; Redgate et al., 2014; van den Berg et al., 2016b). Such studies demonstrate that horses can discriminate between diets based on both energy and CP content, even if foods are novel and regardless of flavour (odour) preferences.

The influence of sweetener and odour on diet selection

Diet preferences due to flavours have not been widely examined in horses (Burton et al., 1983; Kennedy et al., 1999; Goodwin et al., 2005a; b) and in these studies it is not always clear what type of flavouring was used; for example non-nutritive vs nutritive, or aromatic vs taste that may have calories or not (sugar versus artificial or natural sweeteners). In the present study a non-caloric (natural) sweetener was used so that a taste effect could be assessed without interfering with the nutritional content. While nutrient content seems to be the primary determinant for diet selection, the results of the two-choice and multiple-choice

testing also suggest that an added taste enhances preference, with a partial preference for LP+ and HP and the highest consumption for HP+.

A recent study has shown that horses express the taste receptor gene T1R2 in lingual epithelium (taste buds) and both T1R2 and T1R3 in intestinal endocrine cells, which play an important role in the sensing of sugars and other sweet compounds (Daly et al., 2012). However, to our knowledge there are no previous equine studies that have reported the use of non-caloric artificial or natural sweeteners in horse diets and that clearly show the positive effects on preferences of taste using non-caloric natural sweeteners. The inclusion of artificial or natural sweeteners to animal diets is a common practice in the swine industry (Munro et al., 2000; Sterk et al., 2008; Moran et al., 2010) where sweeteners are routinely included in piglet diets to enhance feed palatability and avoid a drop in feed intake post-weaning. However, there are somewhat variable results of the effect of sweetener on feed intake, feed conversion and daily weight gain in piglets; showing positive effects when an artificial sweetener (Sucram) was used (Sterk et al., 2008), whereas the natural sweetener Stevia did not appear to have detrimental effects on feed consumption and performance of piglets (Munro et al., 2000). It is well known that stevia can have a bitter aftertaste in humans (Goyal et al., 2010), which could explain why stevia may not be as useful in enhancing palatability. In our study we used a blend of erythritol and stevia (with erythritol being the bulk sweetener), which reduces the bitter aftertaste of stevia and provides an equal sugar (1:1) sensation (de Cock, 2012). As a bulk sweetener, erythritol provides volume, texture and microbiological stability similar to sucrose. In addition, quantitative descriptive analysis shows that erythritol solutions taste similar to sucrose (de Cock, 2012) and therefore may be more effective in enhancing palatability. While this study showed the positive effect of a blend of erythritol and stevia on diet preference, further research is needed that tests the effect of different (pure and blended)

natural and artificial sweeteners on the food palatability and voluntary feed intake by horses. This could provide new insight in useful additives for the horse feed industry.

While nutrients and taste seem to have a greater influence on diet intake, our study was also able to show that an aromatic flavour (odour) can affect intake. When assessing both preference tests, a greater intake was recorded for diets linked to the banana odours followed by coconut. This pattern is in accordance with the results of Goodwin et al. (2005a), who also ranked banana flavouring as most preferred of the 15 flavours. These findings suggest that horses have a preference for odours that can be described as having a sweet aromatic sensation, even when not linked to nutritive characteristics.

Multiple-choice test model to simulate patch foraging conditions

In a natural or grazing environment horses select from a diverse range of resources, which suggests that multiple-choice tests may be advantageous when assessing preferences. In the present study a chequerboard 'patch' design was used, which clearly demonstrated that horses select from all foods but have ranked preferences associated with macronutrients, taste then odour. This ranking was also identified in the contrast tests based on the mean intake of the diets, but was less obvious when two diets were compared (contrasts). It seems that a patch design was the most appropriate for pasture field studies that reviewed the preference for short and tall sward heights (Naujeck et al., 2005; Edouard et al., 2009; Edouard et al., 2010). Other equine studies (Goodwin et al., 2002; Thorne et al., 2005; Goodwin et al., 2007) have used a multiple choice design to assess the intake and feeding behaviour of stabled horses and demonstrated that horses selected from preferred and less preferred forages, evidently mixing diets. Goodwin et al. (2007) also showed that horses moved between forage locations regardless of the palatability of the forages or horse's preference for a particular forage

indicating that searching/ patch foraging behaviour is an important component in diet selection by horses.

In the present study, searching behaviour, i.e. time spent moving towards the buckets/ zones and the visits to each bucket/zone, was assessed in both the two-choice and multiple-choice test. No differences in the ratios for bucket visits and time spent moving between days and groups were recorded for the two-choice testing. In addition, the results showed a close to 1:1 ratio for time spent moving and bucket visits for all tests. In the multiple-choice test horses did spent significantly more time moving towards the HP and HP+ diets compared to the LP and LP+ diets. However no differences in the mean zone count between diets were observed. The equal zone count suggests that horses displayed continuous sampling behaviour and possibly did not appear to use spatial cues to identify preferred patches/ zones. This confirms the findings of a previous study (van den Berg et al., 2016a). It has been suggested that grazing animals may rely more on visual or orosensory cues rather than on memory of spatial cues when faced with a heterogeneous environment (unpredictability) and depending on the spatial and temporal scale of the foraging hierarchy (Illius and Gordon, 1990; Hewitson et al., 2005). Hewitson et al. (2005) demonstrated that sheep can use spatial cues on the smaller spatial scales (feeding site or patch) to improve foraging efficiency where resource distribution was predictable, but when feed position became less predictable animals increased sampling behaviour, which suggests that grazing animals can switch between foraging tactics. In this study, where feed bucket positions were daily randomised, the motivation to move from one patch to another can therefore be related to sampling behaviour (trial and error), which allows animals to get information about the sensory characteristics that animal's link to the nutritional consequences of foods (olfactory memory).

Group effect

A strong group effect was observed for both the two-choice and multiple-choice tests with Group B showing a significantly greater preference for the diets with greater palatability (higher contrast) compared to the other groups in the two-choice contrast tests. This was linked with the lowest overall mean intake and was similar for both test protocols. This group also spent less time moving and had the lowest mean zone count, which makes this group of horses more selective in terms of feed choices. It is unclear why this group displayed such differences as the groups were randomly allocated based on age, weight and sex. The age of the group ranged from 4 to 14, showing a similar age distribution as Group A and C. Group D had a lower average age, however like Group B had 1 male horse and 3 female horses. In addition, during the adaptation phase both Group B and D showed similar variance in diet intake. Therefore these results may simply reflect individuality and highlight that there may be large variation between animals in how they regulate intake of nutrients to meet dietary needs. Further studies that integrate nutritional geometry models could gain more insight in these regulatory mechanisms of individuals. In a geometric framework for nutrition, the important components of animal nutrition (e.g. foods, nutrient requirements, nutrient utilisation) are defined in a Cartesian space, where each dimension represents a food constituent (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993). While these frameworks have been extensively studied in various insect and vertebrate species, at present no studies have been conducted with horses (Raubenheimer and Simpson, 1997). This highlights the opportunity to integrate these geometric models to answer some of the more complex questions as to how (individual) horses use nutrient intake targets to regulate feed intake given a number of choices.

Conclusion

This study was able to show that horses sample all diets on offer but show clear preferences ranked on nutrients, followed by taste then odour. This ranking was more evident in the multiple-choice testing than the two-choice testing and suggests that a multiple-choice model such as a chequerboard design could be more informative when ranking preferences. However, an adaptation period is needed to allow for post-ingestive associations. Further research is required to assess the use of these types of preference models in natural or pasture environments. While our study is in accordance with other research showing that nutrients have a strong influence on diet selection, we should also acknowledge the importance of taste and odour on diet selection. To our knowledge this is the first study that has been able to show the positive effects of a non-caloric natural sweetener (erythirol and stevia blend) on diet intake and selection. This new knowledge could be useful for enhance palatability in equine diets, without affecting the glycaemic index. However, further studies are needed that evaluate different types of sweeteners coupled with and without odour and/or dietary nutrients and its long-term effects on food intake by horses.

Conflict of interest

Funding for this project was kindly provided by the University of New England, New South Wales, Australia. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no additional financial support for this work that could have influenced its outcome.

Ethical statement

The care and use of the animals followed the guidelines set by The University of New England Animal Ethics Committee, in accordance with section 25 of the Animal Research Act (1985).

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Table 1. Chemical composition[a] (g/kg dry matter (DM)) of the four diets (LP; low protein, LP+; low protein + sweetener, HP; high protein, and HP+; higher protein + sweetener) offered to horses (n=16) during the feeding trial.

Table 2. Four treatment diets and associated odours for each group of horses (n = 4) in a 4 x 4 Latin Square design.

Table 3. Sixteen adult horses were paired based on weight, age and sex (mare (M) and gelding (G)) and randomly allocated to one of the four treatment groups to create even animal group characteristics.

Table 4. Phase 2: Two-choice test. Diets were paired based on contrast to examine preferences and diet ranking. (LP; low protein, LP+; low protein + sweetener, HP; high protein, and HP+; higher protein + sweetener).

Table 5. GLM (binomial) parameters fitted to ratios of intake, bucket visits and time spent foraging or moving of lower (Bucket 1) to higher (Bucket 2) palatability contrast for the four two-choice tests (16 horses; n=4 per group). The fitted parameters (± SE) of the GLM model with the (back-transformed) expected ratios are presented.

Table 6. Linear regression and GLM (Poisson) parameters (± SE) fitted to intake, zone count and time spent foraging or moving for the multiple-choice test (16 horses; n=4 per group). Intake and time spent foraging are based on linear regression models. For time spent moving and zone count fitted parameters of the GLM models with the (back-transformed) expected means are presented.

Figure 1. Timeline of the experiments. Phase 1 was the adaptation phase to establish flavour-to-post-ingestive associations (LP; low protein diet, LP+; low protein diet + sweetener, HP; high protein diet and HP+; high protein diet + sweetener). Phase 2 was the two-choice contrast tests (LP v.s. LP+, LP v.s. HP, HP v.s. HP+ and LP+ v.s. HP+). Phase 3 was the multiple-choice test using a checkerboard design (Smörgåsbord).

Figure 2. Field and patch layout. A testing area (12 m x 12 m) divided into 16 zones (2.5 m^2). There were 4 zones allocated to each odour/diet combination in a chequerboard fashion. On every testing day the diets were randomly allocated to a new zone. Horses (n=16) were individually led into the testing area and allowed 7.5 minutes to forage the area uninhibited, which was recorded with video recorders and by direct observation.

Figure 3. Feed intake of each diet over the four weeks (adaptation phase) was assessed to determine the acceptance of the diets and post-ingestive associations. For illustration purposes the proportion (%) and trends (line) of diet intake on the logit scale 0-100% (min; -15 to max; 15) over 5 test days was selected (n=16 horses). Logit of 1.4 is equal to 80% feed intake. LP; low protein, LP+; low protein + sweetener, HP; high protein, and HP+; higher protein + sweetener.

Table 1. Chemical composition[a] (g/kg dry matter (DM)) of the diets (LP; low protein, LP+; low protein + sweetener, HP; high protein, and HP+; higher protein + sweetener) offered to horses (n=16) during the feeding trial.

Constituent	LP	LP+	HP	HP+
Dry Matter	903	902	920	925
Digestible Energy (MJ/kg DM)	12.7	12.9	12.4	12.5
Crude Protein	140	141	266	270
NDF	334	312	325	306
ADF	212	209	219	203
NFC	431	451	314	327
Starch	277	249	145	144
WSC	58	58	50	48
ESC	43	33	25	31
Calcium	3.5	3.6	4.1	3.6
Phosphorus	2.3	2.7	2.7	3.0
Magnesium	1.7	1.8	1.5	1.5
Potassium	6.7	6.8	6.4	5.9

[a] NDF, neutral detergent fibre; ADF, acid detergent fibre; NFC, non-fibre carbohydrates, WSC; water soluble carbohydrates, ESC; ethanol soluble carbohydrates. Units are g/kg DM, unless otherwise stated.

Table 2. Four treatment diets and associated odours for each group of horses (n = 4) in a 4 x 4 Latin Square design.

Protein	Sweetener		Group A	Group B	Group C	Group D
Low	-	LP	Coconut	Cinnamon	Spearmint	Banana
Low	+	LP+	Banana	Coconut	Cinnamon	Spearmint
High	-	HP	Spearmint	Banana	Coconut	Cinnamon
High	+	HP+	Cinnamon	Spearmint	Banana	Coconut

Table 3. Sixteen adult horses were paired based on weight, age and sex (mare (M) and gelding (G)) and randomly allocated to one of the four treatment groups to create even animal group characteristics.

	Group A			Group B			Group C			Group D		
	Weight	**Age**	**Sex**	**Weight**	**Age**	**Sex**	**Weight**	**Age**	**Sex**	**Weight**	**Age**	**Sex**
Horse 1	516	15	M	528	4	M	520	4	G	530	12	G
Horse 2	538	6	G	532	12	G	548	12	G	538	5	M
Horse 3	582	7	M	578	14	M	578	12	M	572	5	M
Horse 4	602	10	G	602	7	M	584	13	M	602	6	M
Mean ± SD	560 ± 39	10 ± 4		560 ± 36	9 ± 5		558 ± 30	10 ± 4		561 ± 33	7 ± 3	

Table 4. Phase 2: Two-choice test. Diets were paired based on contrast to examine preferences and diet ranking.

Test	Choice 1	Choice 2
1	LP	LP+
2	LP	HP
3	HP	HP+
4	LP +	HP+

(LP; low protein, LP+; low protein + sweetener, HP; high protein, and HP+; higher protein + sweetener)

Table 5. GLM (binomial) parameters fitted to ratios of intake, bucket visits and time spent foraging or moving of lower (Bucket 1) to higher (Bucket 2) palatability contrast for the four two-choice tests (16 horses; n=4 per group). The fitted parameters (± SE) of the GLM model with the (back-transformed) expected ratios are presented.

a) Log-ratio Intake

Test	Intercept	Day 2	Day 3	Group B	Group C	Group D	*P* (Day)	*P* (Group)
1: LP vs. LP+	-0.068 ± 0.039 (0.93)	0.086 ± 0.039 (× 1.09)	0.1 ± 0.039 (× 1.11)	-0.098 ± 0.046 (× 0.91)	0.009 ± 0.044 (× 1.0)	0.009 ± 0.044 (× 1.0)	0.02	0.051
2: LP vs. HP	-0.07 ± 0.039 (0.93)	-0.034 ± 0.039 (× 0.97)	0.036 ± 0.039 (× 1.04)	-0.11 ± 0.047 (× 0.9)	0.044 ± 0.044 (× 1.05)	0.059 ± 0.044 (× 1.06)	NS	<0.001
3: HP vs. HP+	-0.043 ± 0.038 (0.96)	0.012 ± 0.039 (× 1.01)	0.034 ± 0.038 (× 1.04)	-0.073 ± 0.045 (× 0.93)	0.023 ± 0.044 (× 1.02)	0.014 ± 0.044 (× 1.01)	NS	NS
4: LP+ vs. HP+	-0.015 ± 0.038 (0.99)	0.018 ± 0.038 (× 1.02)	0.004 ± 0.038 (× 1.0)	-0.149 ± 0.045 (× 0.86)	-0.028 ± 0.043 (× 0.97)	-0.012 ± 0.044 (× 0.99)	NS	0.003

b) Log-ratio Time spent foraging

Test	Intercept	Day 2	Day 3	Group B	Group C	Group D	*P* (Day)	*P* (Group)
1: LP vs. LP+	-0.082 ± 0.043 (0.92)	0.158 ± 0.045 (× 1.17)	0.247 ± 0.044 (× 1.28)	-0.217 ± 0.05 (× 0.81)	-0.037 ± 0.05 (× 0.96)	-0.041 ± 0.05 (× 0.96)	<0.001	<0.001
2: LP vs. HP	-0.151 ± 0.042 (0.86)	-0.024 ± 0.043 (× 0.98)	0.004 ± 0.043 (× 1.0)	-0.273 ± 0.049 (× 0.76)	0.111 ± 0.05 (× 1.12)	0.053 ± 0.049 (× 1.05)	NS	<0.001
3: HP vs. HP+	-0.105 ± 0.043 (0.9)	0.244 ± 0.044 (× 1.28)	0.055 ± 0.043 (× 1.06)	-0.1 ± 0.049 (× 0.91)	0.138 ± 0.051 (× 1.15)	0.089 ± 0.051 (× 1.09)	<0.001	<0.001
4: LP+ vs. HP+	0.175 ± 0.043 (1.19)	0.045 ± 0.044 (× 1.05)	-0.23 ± 0.044 (× 0.79)	-0.449 ± 0.05 (× 0.64)	-0.13 ± 0.051 (× 0.88)	-0.137 ± 0.051 (× 0.87)	<0.001	<0.001

c) Log-ratio Time spent moving

Test	Intercept	Day 2	Day 3	Group B	Group C	Group D	*P* (Day)	*P* (Group)
1: LP vs. LP+	-0.201 ± 0.185 (0.82)	0.005 ± 0.177 (× 1.01)	0.209 ± 0.185 (× 1.23)	0.149 ± 0.201 (× 1.16)	0.198 ± 0.184 (× 1.22)	0.062 ± 0.187 (× 1.06)	NS	NS
2: LP vs. HP	-0.162 ± 0.215 (1.18)	-0.119 ± 0.21 (× 0.89)	-0.052 ± 0.22 (× 0.95)	-0.356 ± 0.243 (× 0.7)	-0.257 ± 0.23 (× 0.77)	-0.234 ± 0.24 (× 0.79)	NS	NS
3: HP vs. HP+	0.192 ± 0.197 (1.21)	-0.252 ± 0.183 (× 0.78)	-0.079 ± 0.184 (× 0.92)	0.033 ± 0.22 (× 1.03)	-0.133 ± 0.205 (× 0.87)	-0.007 ± 0.209 (× 0.99)	NS	NS
4: LP+ vs. HP+	0.115 ± 0.221 (1.12)	-0.394 ± 0.203 (× 0.67)	0.033 ± 0.202 (× 1.03)	0.073 ± 0.25 (× 1.08)	0.075 ± 0.231 (× 1.08)	0.03 ± 0.25 (× 1.03)	0.059	NS

d) Log-ratio Bucket visits

Test	Intercept	Day 2	Day 3	Group B	Group C	Group D	*P* (Day)	*P* (Group)
1: LP vs. LP+	-0.035 ± 0.267 (0.97)	-0.103 ± 0.257 (× 0.9)	0.115 ± 0.272 (× 1.12)	0.118 ± 0.316 (× 1.13)	0.082 ± 0.285 (× 1.09)	0.102 ± 0.287 (× 1.11)	NS	NS
2: LP vs. HP	0.106 ± 0.324 (1.11)	-0.158 ± 0.315 (× 0.85)	0.07 ± 0.316 (× 1.07)	-0.243 ± 0.378 (× 0.78)	-0.081 ± 0.365 (× 0.92)	-0.104 ± 0.367 (× 0.9)	NS	NS
3: HP vs. HP+	0.12 ± 0.266 (1.13)	-0.081 ± 0.26 (× 0.92)	-0.062 ± 0.258 (× 0.94)	-0.067± 0.319 (× 0.94)	-0.09 ± 0.291 (× 0.91)	0.005 ± 0.297 (× 1.0)	NS	NS
4: LP+ vs. HP+	0.013 ± 0.304 (1.01)	-0.159 ± 0.295 (× 0.85)	0.095 ± 0.297 (× 1.1)	0.098 ± 0.385 (×1.1)	0.072 ± 0.335 (× 1.07)	0.04 ± 0.355 (× 1.04)	NS	NS

LP; low protein, LP+; low protein + sweetener, HP; high protein, and HP+; higher protein + sweetener

NS: Not significant

All models had 48 observations (Residual df. 45 (Day) and 42 (Group)).

Table 6. Linear regression and GLM (Poisson) parameters (± SE) fitted to intake, zone count and time spent foraging or moving for the multiple-choice test (16 horses; n=4 per group). Intake and time spent foraging are based on linear regression models. For time spent moving and zone count fitted parameters of the GLM models with the (back-transformed) expected means are presented.

	Intake (g, DM)	**Time spent foraging** (sec)	**Time spent moving** (log-mean; (sec))	**Zone count** (log-mean; (count))
Intercept	109.3 ± 15	89.6 ± 11.2	2.2 ± 0.07 (8.8)	0.99 ± 0.13 (2.7)
Diet LP+	40.4 ± 11.3	22.5 ± 8.5	0.01 ± 0.06 (× 1.01)	0.05 ± 0.1 (× 1.05)
Diet HP	41.5 ± 11.3	29.6 ± 8.5	0.15 ± 0.06 (× 1.16)	0.16 ± 0.1 (× 1.18)
Diet HP+	73.6 ± 11.3	44.6 ± 8.5	0.12 ± 0.06 (× 1.13)	0.14 ± 0.1 (× 1.15)
Day 2	20.1 ± 12.6	10.7 ± 9.5	-0.04 ± 0.07 (× 0.96)	0.09 ± 0.11 (× 1.09)
Day 3	15.9 ± 12.6	9.1 ± 9.5	0.01 ± 0.07 (× 1.01)	0.08 ± 0.11 (× 1.08)
Day 4	11.4 ± 12.6	6.4 ± 9.5	0.01 ± 0.07 (× 1.01)	0.03 ± 0.11 (× 1.03)
Day 5	18.1 ± 12.6	8.1 ± 9.5	0.17 ± 0.06 (× 1.18)	0.21 ± 0.11 (× 1.23)
Odour Cinnamon	-34.7 ± 11.3	-35.2 ± 8.5	-0.06 ± 0.06 (× 0.94)	-0.09 ± 0.1 (× 0.91)
Odour Coconut	-20.6 ± 11.3	-18.8 ± 8.5	-0.03 ± 0.06 (× 0.97)	-0.04 ± 0.1 (× 0.96)
Odour Spearmint	-55.0 ± 11.3	-41.9 ± 8.5	-0.26 ± 0.06 (× 0.77)	-0.21 ± 0.1 (× 0.81)
Group B	-20.3 ± 11.3	5.9 ± 8.5	-0.49 ± 0.06 (× 0.61)	-0.48 ± 0.11 (× 0.62)
Group C	45.8 ± 11.3	4.4 ± 8.5	-0.02 ± 0.05 (× 0.98)	0.01 ± 0.09 (× 1.01)
Group D	50.9 ± 11.3	4.3 ± 8.5	-0.18 ± 0.06 (× 0.84)	-0.07 ± 0.09 (× 0.93)
***P* (Diet)**	P<0.001	P<0.001	P=0.013	NS
***P* (Day)**	NS	NS	P=0.009	NS
***P* (Odour)**	P<0.001	P<0.001	P<0.001	NS
***P* (Group)**	P<0.001	NS	P<0.001	P<0.001

LP; low protein, LP+; low protein + sweetener, HP; high protein, and HP+; higher protein + sweetener

NS: Not significant

320 observations (Residual df. 316 (Diet), 312 (Day), 309 (Odour) and 306 (Group)).

Figure1-Timeline-experiments

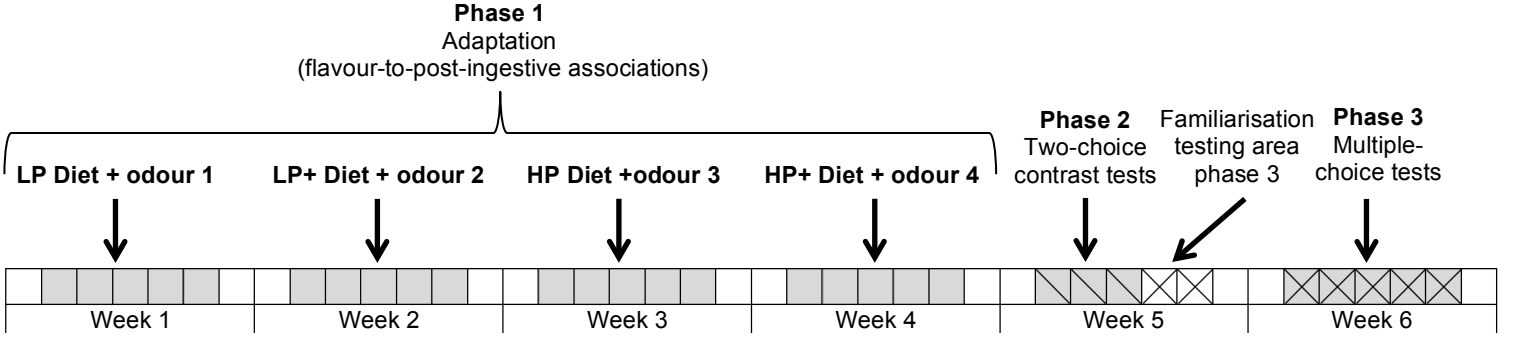

Figure2-Design-Testing-Area-Phase3

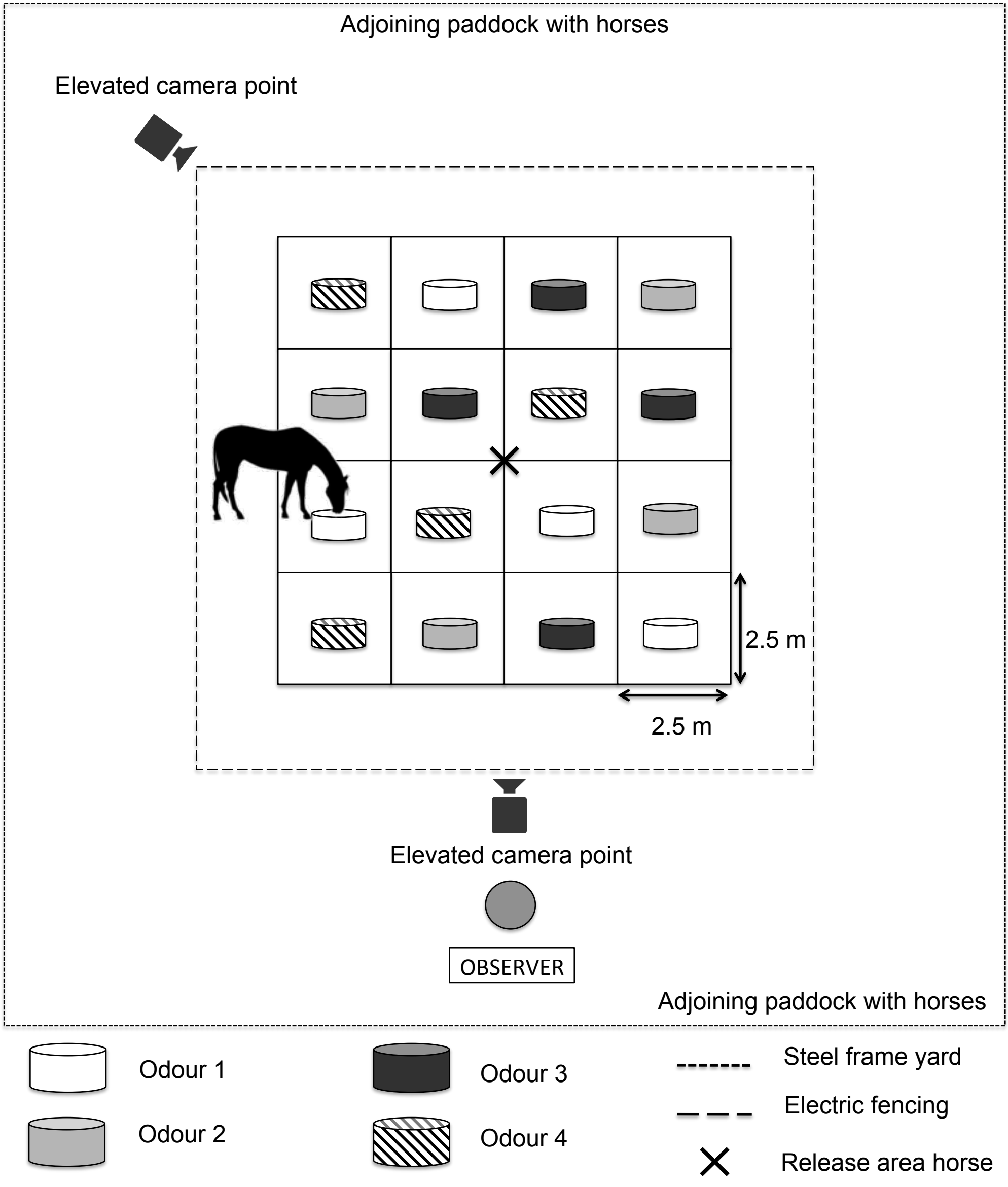

Figure3-Phase1-Diets-xyplot